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Contact Inhibition: Also a Control for Cell Proliferation in Unicellular Algae?

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Abstract. According to traditional views, the proliferation of unicellular algae is controlled primarily by environmental conditions. But as in mammalian cells, other biological mechanisms, such as growth factors, cellular aging, and contact inhibition, might also control algal proliferation. Here we ask whether contact inhibition regulates growth in several species of unicellular algae as it does in mammalian cells. Laboratory cultures of the dinoflagellate *Prorocentrum lima* (Ehrenberg) Dodge show contact inhibition at low cell density, so this would be an autocontrol mechanism of cell proliferation that could also act in natural populations of *P. lima*. But, *Synechocystis spp.*, *Phaeodactylum tricornutum* (Bohlin), *Skeletonema costatum* (Greville), and *Tetraselmis spp.* do not exhibit contact inhibition in laboratory cultures because they are able to grow at high cellular density. Apparently their growth is limited by nutrient depletion or catabolite accumulation instead of contact inhibition. *Spirogyra insignis* (Hassall) Kutz, *Prorocentrum triestinum* Schiller, and *Alexandrium tamarense* (Halim) Balech show a complex response, as they are able to grow in both low and high cell density medium. These results suggest that contact inhibition is more adaptative in benthic unicellular algae.

Introduction

Environmental conditions (light, nutrients, temperature, and turbulence) are thought to be the main controls of proliferation in unicellular algae. Thus, axenic cultures of algae progressively increase in cell number until division slows due to nutrient depletion, the shadowing of some cells by others, or metabolite accumulation. But other mechanisms could play an important role in autocontrol of algal proliferation. In this respect, endogenous rhythms have been proposed as pacemakers of algal proliferation (reviewed by Edmunds, 1988). Also, mucilage production has been considered a mechanism of biological autocontrol in unicellular algae (Margalef, 1989). Recently, Wyatt and Reguera (1989) proposed that the onset of phytoplankton blooms and red tides are due to a mechanism of ecological autocontrol acting at the Gaian level.

Several biological mechanisms that control the cell division cycle in mammalian cells have recently been elucidated. They are based on growth factors, genes, and gene products that respond to growth factors (Baserga *et al.*, 1986; Goustin *et al.*, 1986; Cantley *et al.*, 1991; North, 1991). Although these mechanisms have been interpreted as adaptations for regulating cellular proliferation in multicellular organisms, they are common to all eukaryotic cells, even regulating the cleavage of zygotes (Murray and Kirschner, 1989). Recently, we have proven that the cell division cycle in unicellular algae from different phyla (Cyanophyceae, Dinophyceae, Bacillarophyceae, and Chlorophyceae) are regulated by growth factors just as are mammalian cells (Costas and López-Rodas, 1991a; López-Rodas *et al.*, 1991).

In addition to regulation by growth factors, other mechanisms control the cell proliferation of mammalian cells. For example, some cells are genetically programmed to degenerate and die of old age after a determined number of generations. Also, the unicellular algae *Spirogyra insignis* (Conjugatophyceae) undergoes cellular aging as do mammalian cells (Costas and López-Rodas, 1991b).

In mammals, another important regulator of cellular proliferation is contact inhibition. Mammalian cells grow in monolayers, colonizing the bottom of culture flasks, but they only increase until their growth is inhibited by contact with neighboring cells. Various mechanisms seem to be involved in this complex phenomenon, from growth

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Table I

Characteristics of the species used

Species	Phyla	Characteristic
Synechocystis spp.	Cyanobacteria	unicellular, planktonic
Prorocentrum lima (Ehrenberg) Dodge	Dinophyceae	unicellular, benthic
Prorocentrum triestinum Schiller	Dinophyceae	unicellular, swimming
Alexandrium tamarense (Halim) Balech	Dinophyceae	unicellular, swimming
Tetraselmis spp.	Praxinophyceae	unicellular, swimming
Skeletonema costatum (Greville)	Bacillarophyceae	cenobial filamentous, planktonic
Phaeodactylum tricornutum (Bohlin)	Bacillarophyceae	unicellular, benthic
*Spirogyra insignis** (Hasak) Kutz	Conjugatophyceae	cenobial filamentous, benthic

* *Spirogyra insignis* grows in cenobial filaments anchored to the bottom of the flask by the distal cell. Every cell of the filament can divide (more detail in Costas and López-Rodas, 1991b).

factor competence to cell shape changes related to intercell contacts (review Alberts *et al.*, 1983).

This paper attempts to determine whether contact inhibition can limit the growth of unicellular algae, as is the case in mammalian cells. Several species of unicellular algae from different phyla are analyzed in a combined ecological and evolutionary approach.

Materials and Methods

Cultures

Isolation and culture procedures for the species used were previously described in detail (Costas, 1990; Costas and López-Rodas, 1991a, b, c), so only a brief description is provided here.

The characteristics of the eight species employed are summarized in Table I. Freshwater and marine species were grown, respectively, in Petri dishes with 20 ml of WC medium or f/2 medium (Guillard, 1975), at 22.5 $\pm$ 0.5°C and 80 μmol m^{-2} s^{-1}, 12:12 h light-dark cycle.

Cultures were treated with 150 mg l^{-1} penicillin and 100 mg l^{-1} streptomycin and were, therefore, axenic. Before the experiments were performed, the cultures were tested for the presence of bacteria using epifluorescence procedures as previously described (Costas, 1990). The possible effects of antibiotics on algal proliferation were obviated, because the antibiotic treatment was applied two months before the experiments took place, so the cultures were grown under axenic conditions.

Cultures were maintained by serial transfers of a 500 $\pm$ 30 cell inoculum to fresh medium once every day. The cells grew exponentially for 20–30 days, and then the cultures showed density-dependent inhibition of growth. We determined that a culture reached saturation when its growth rate approached zero and its cell density reached the maximum. Saturation was easily detected because, growth rates and cell densities were determined daily. The experiments took place three days after the cultures were saturated.

Experimental Design

Many factors act in the cell density-dependent inhibition of growth. In this investigation, we attempted to analyze whether contact inhibition also takes part in this process. Clonal cultures of each species were grown until saturation density was reached, and then the following two experiments were performed.

Experiment 1: Cells at saturation density growing in fresh medium. All the cells of each saturated culture were collected (by centrifugation at 1000 rpm for 20 min), and resuspended in the same quantity of fresh medium. In this way we obtained a culture in fresh medium with saturated density of cells. Growth rates and cellular densities were measured during the five following days. Five replicates were performed for each species.

Experiment 2: Cells at low density growing in saturated medium. In the second experiment, the saturated medium, after centrifugation, was filtered through a 0.22 μm pore filter to produce a completely axenic, saturated medium that was free of cells. In this saturated, cell-free medium, a centrifuged inoculum of the same species growing exponentially, was cultured. Growth rates and cellular densities were measured during the five following days. Five replicates were performed for each species.

If inhibition of growth by contact inhibition and other factors are mutually exclusive, then contact inhibition of growth can be detected by this system, according to the following logic. If a species exhibits contact inhibition, then it will probably be able to grow in Experiment 2, but it won't be able to proliferate in Experiment 1. On the contrary, if the growth inhibition is due to other factors (nutrient depletion or catabolite accumulation), then it will probably be able to grow in Experiment 1 but not in Experiment 2. But, if other factors (*i.e.*, soluble factors), as well as contact inhibition affect growth, then the simple two possibility choice won't happen.

To determine whether contact inhibition is a factor in growth inhibition of those algae that grow in monolayers, the following experiment was performed; *i.e.*, the same method used to detect contact inhibition in mammalian cells was applied to algae. The cells from half a Petri dish were removed mechanically from each saturated culture

Table II

Growth rates and percentage increase of cell density in fresh medium at saturation density and in saturated medium at low density

	Exponential growth-rates	Saturated growth-rates	Cells at saturation density in fresh medium		Cells at low density in saturated medium	
			Growth rates	% increase cell density	Growth rates	% increase cell density
Synechocystis spp.	0.79 ± 0.01	−0.07 ± 0.006	0.49 ± 0.06**	64 ± 5%	−0.04 ± 0.01**	−4 ± 1%
Prorocentrum lima	0.38 ± 0.03	0.01 ± 0.01	0.03 ± 0.02**	2 ± 1%	0.39 ± 0.07**	47 ± 2%
Prorocentrum triestinum	0.91 ± 0.05	0.01 ± 0.01	0.29 ± 0.02**	33 ± 1%	0.07 ± 0.02**	7 ± 2%
Alexandrium tamarense	0.43 ± 0.03	−0.03 ± 0.02	0.12 ± 0.05*	12 ± 5%	0.07 ± 0.02*	7 ± 1%
Tetraselmis spp.	0.96 ± 0.05	−0.04 ± 0.01	0.87 ± 0.07**	138 ± 6%	0.01 ± 0.01**	1 ± 1%
Skeletonema costatum	1.01 ± 0.07	−0.02 ± 0.03	0.58 ± 0.03**	78 ± 3%	−0.07 ± 0.01**	−6 ± 1%
Phaeodactilum tricornutum	0.88 ± 0.04	0.02 ± 0.01	0.52 ± 0.01**	68 ± 2%	−0.03 ± 0.02**	−2 ± 3%
Spirogyra insignis	0.94 ± 0.05	0.03 ± 0.02	0.18 ± 0.05**	19 ± 4%	0.68 ± 0.05**	97 ± 6%

* Statistically no significant differences were found ($P > 0.05$).

** Statistically significant differences ($P < 0.01$) were found between growth rates of Exp. 1 and Exp. 2.

sample of monolayer species. If contact inhibition exists, the cells on the full side will continue growing into the cell-free half of the dish. Five replicates were performed in each case. A continuous recording by video microscopy helped us to evaluate this experiment.

Control of handling effects

Because some dinoflagellates are very sensitive to shear stress, we performed the following two preliminary experiments to determine whether manipulation would have detectable effects on the analyzed species.

(a) Exponentially growing cells of each species were collected by centrifugation at 1000 rpm for 20 min and resuspended in the same quantity of fresh medium. Their growth rates (5 replicates of each species) were measured during the following five days and compared with the growth rates of uncentrifuged exponentially growing controls (5 replicates of each species). ANOVA analysis showed no significant differences ($P > 0.05$) between growth rates of centrifuged and uncentrifuged cells. Furthermore, the number of dead cells was estimated by the yellow eosine exclusion procedure (more details in Costas, 1986; González-Chavarri, 1991), and ANOVA showed no significant differences ($P > 0.05$) in the rate of cell death between centrifuged and uncentrifuged cells.

(b) A similar procedure was employed with saturated cells, and the same results were obtained; (*i.e.*, there were no significant differences ($P > 0.05$) between centrifuged and uncentrifuged cells). More details about the procedures used to control the effects of handling are set out in Costas (1986) and González-Chavarri (1991).

Table III

Growth rates of Prorocentrum lima *and* Spirogyra insignis *after cells were mechanically removed from half a Petri dish*

	Border where cells had been removed	Zone where cells had not been removed	F
Prorocentrum lima	0.31 ± 0.07	0.01 ± 0.01	$P < 0.01$
Spirogyra insignis	0.47 ± 0.03	0.13 ± 0.02	$P < 0.01$

Experimental evaluation

Once an experiment was initiated for each of the five replicates, both the mean growth rates (during the subsequent five days) and the percentage of cell density increase (during the subsequent 24 h) were determined. Cell density was estimated as the number of cells per square or cubic centimeter in monolayer or suspension cultures, respectively. The number of cells in each culture was determined by counting samples in a hemocytometer. The number of samples counted was determined according to the mean progressive technique (Williams, 1977) to obtain 95% accuracy.

Growth rates were calculated as doublings per day:

$$dd^{-1} = 1/\mathrm{Ln}2\ \mathrm{Ln}(Nt/No)/t,$$

Where Nt = cells at time t; No = cells at time 0; and t = number of days between times t and 0 (more detail in Costas, 1990).

Results and Discussion

Growth inhibition of saturated cultures of unicellular algae is a complex process, influenced by various factors, such as nutrient depletion, catabolite accumulation, shading effects, and possibly by contact inhibition. Because these factors do not act independently, their inter-

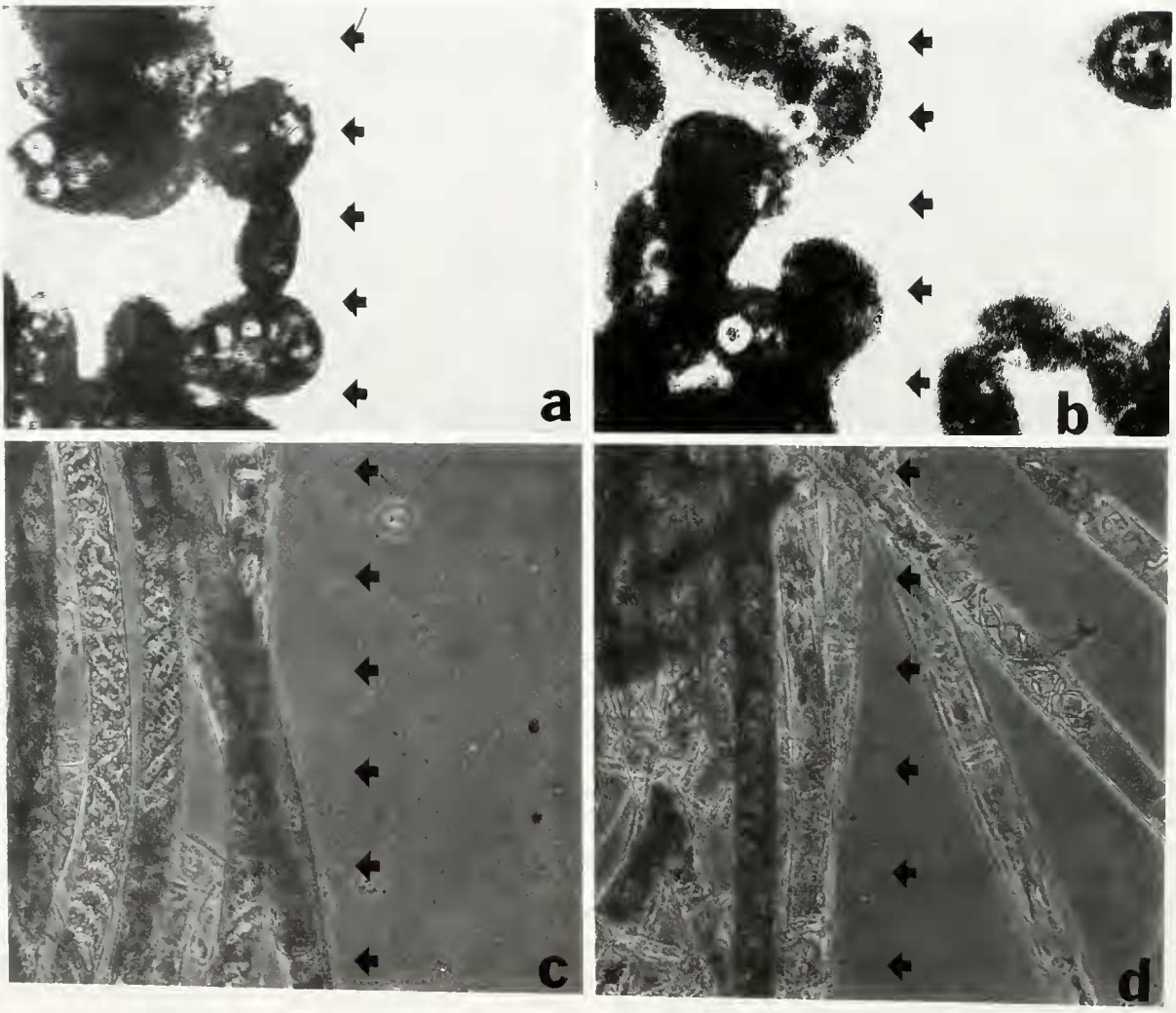

Figure 1. Growth of *Prorocentrum lima* and *Spirogyra insignis* when cells were mechanically removed from half a Petri dish. The arrows represent the border produced in the experiment. Only the cells bordering the cell-free zone were able to grow. (a) Saturated *P. lima* culture at the time of removal. (b) *P. lima* culture 72 h after the removal. New cells have only proliferated into the open half of the plate. (c) Saturated *S. insignis* culture at the time of removal. (d) *S. insignis* culture 72 h after the removal. New cells have only proliferated into the free half of the plate.

actions complicate a precise evaluation of the relative importance of each. Thus, our experimental design was aimed only at detecting whether contact inhibition takes part in cell dependent inhibition of growth.

Table II summarizes the growth rates and the percentage of cell density increases in both fresh and saturated culture media. Apparently, the dinoflagellate *P. lima* showed contact inhibition of growth. Both the growth rates and the cell densities of Experiments 1 and 2 were significantly different ($P < 0.01$). *P. lima* cells were not able to grow at saturation density in fresh medium (Experiment 1), but their growth started again in saturated medium when their cell density decreased (Experiment 2).

In contrast, *Synechocystis spp., Phaeodactilum tricornutum, Skeletonema costatum* and *Tetraselmis spp.* did not exhibit contact inhibition. In all the cases, statistically significant differences ($P < 0.01$) were detected between both the growth rates and the cell densities of Experiments 1 and 2. Apparently, their growth was limited by nutrient depletion or catabolite accumulation; thus they could proliferate at high cellular density in fresh medium (Experiment 1), but were not able to grow in saturated medium at low cell density (Experiment 2).

Contact inhibition of growth may be an important mechanism in *Spirogyra insignis*. Although this species grew slowly at saturation density in fresh medium (Ex-

periment 1), its growth was significantly increased ($P > 0.01$) at low density in saturated medium (Experiment 2). So, in *S. insignis*, the contact inhibition component seems to prevail because proliferation is faster in a saturated medium with low cell density than in fresh medium with high cell density.

In *Prorocentrum triestinum*, however, a nutrient dependent inhibition or catabolite accumulation seemed to be more important than contact inhibition. *P. triestinum* was able to grow in both experiments, although its growth in fresh medium at high cellular density was significantly ($P > 0.01$) faster than that in saturated medium at low cell density. In *Alexandrium tamarense*, all of the factors seemed to slow down proliferation. *A. tamarense* cells were scarcely able to grow in either experiment.

The cells of *P. lima* and *S. insignis* were mechanically removed from half a Petri dish, and the resulting growth rates are summarized in Table III. In agreement with previous experiments, the growth of *P. lima* and *S. insignis* seemed to be inhibited by a contact inhibition mechanism. In particular, only the cells bordering the cell-free zone were able to grow (Fig. 1). This experiment, which employs the traditional method of detecting contact inhibition in mammalian cells (Alberts *et al.*, 1983), supports the hypothesis that contact inhibition takes place in the growth inhibition of *P. lima* and *S. insignis* saturated cultures.

Only two of the three benthic species analyzed seemed to exhibit contact inhibition. These results suggest that contact inhibition is a more adaptative mechanism in benthic unicellular algae.

Contact inhibition is usually thought of as a mechanism developed by animal cells to limit cell division. The results obtained in these experiments suggest an alternative interpretation. The dinoflagellates, which could be considered the earliest group of protist, but which are also far removed from actual eukaryotes (Dodge, 1955; Herzog *et al.*, 1984; Costas and Goyanes, 1988), have developed contact inhibition, thereby suggesting that such a mechanism had already been developed by unicellular organisms in an early era, probably as an autocontrol mechanism regulating natural populations. Nevertheless, contact inhibition has also evolved in the Conjugatophyceae (a recent group of higher algae that are phylogenetically far removed from dinoflagellates), suggesting that such mechanisms may have been developed independently in phylogenetically different groups of unicellular organisms.

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